

Integration of morphologic and genetic data clarifies the evolution and species boundaries within a *Nychiodes* Lederer, 1853 species complex (Lepidoptera, Geometridae)

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Abstract

Young species radiations are valuable from an evolutionary perspective, as they can reveal traits involved in species formation, but they are challenging for taxonomists due to discordance among independent lines of evidence. The present study integrates morphologic, ecological, and phylogenetic data to resolve delimitation of species within the geometrid moth genus *Nychiodes* Lederer, 1853, with a particular focus on the closely related species pair *N. divergaria* Staudinger, 1892 and *N. subvirida* Brandt, 1938. Our findings revealed significant ecological niche differentiation across the Iranian mountain ranges between the range of taxa distinguished by morphology and DNA barcodes. While individual gene trees from mtCOI and nuclear loci showed conflicting results regarding species boundaries, our multispecies coalescent analyses support the distinction of taxa with different female genitalia characters. Our study highlights how the combination of morphological, genetic, and ecological data helps to define the boundaries between closely related species, to solve taxonomic uncertainties in regions with a complex evolutionary background.

Key words: DNA barcoding, ecological niche modeling, integrative taxonomy, multispecies coalescent analysis, phylogenetic analysis, species delimitation



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Introduction

The use of morphological characters has traditionally been a cornerstone in taxonomy for the identification and delimitation of insect species (e.g. Cook et al. 2010). Initially, taxonomists relied on external morphological characters, which provided sound criteria for delimiting well-divergent insect taxa. However, these traits were often insufficient for differentiating closely related species or genera until genitalia structures were incorporated, revealing additional crucial diagnostic characters (e.g. Pierce 1914; Hardwick 1950; Sihvonen 2001; Wipfler et al. 2016). While such internal genitalia characters can reveal how selection promotes species formation, e.g., through sexual selection, their interpretation can be complicated by recent divergence times between sister

species, evolutionary constraints due to their core role in reproduction, or because of selection in such traits that may distort the inferred evolutionary history of the diverging species (Smith et al. 2011; Hench et al. 2022). Integrative taxonomy, which consider different taxonomic criteria, such as internal morphological characters, independent genetic markers, and ecological features of their distribution, is an important tool to overcome these challenges towards an understanding of species boundaries and their evolution.

Among these, mitochondrial DNA barcoding of COI has been particularly useful as a rapid and cost-efficient molecular tool for species identification (Rajaei 2015; Antil et al. 2023; Yang et al. 2024). Moreover, these barcodes are collected and curated in online libraries (such as BOLD, <https://boldsystems.org/>), serving as reference databases that assist with species discovery and monitoring (Antil et al. 2023; Yang et al. 2024). Because barcode genes, such as COI, tend to exhibit higher divergence between species than within species, they provide a rapid and reliable approach for species identification, even in cases where morphological differences are subtle, while acknowledging that some intraspecific variation may be present (Hebert et al. 2003a; Kress and Erickson 2008). Nevertheless, DNA barcoding has inherent limitations. Its effectiveness depends on a comprehensive reference database, which often lacks entries for rarely collected or newly described species (Hebert et al. 2003b). Moreover, several genetic and evolutionary factors, such as pseudogenes, intraspecific variation, and introgressive hybridization, complicate species delimitation by causing phylogenetic mismatches between morphological and molecular data (Maddison 1997; Meyer and Paulay 2005; Rubinoff et al. 2006; Song et al. 2008; Degnan and Rosenberg 2009; Yang et al. 2024). These challenges are particularly significant in groups characterized by large population sizes and recent diversification events, where incomplete lineage sorting (ILS), and therefore discordance among independent gene trees, is more common. In Lepidoptera taxonomy, COI based DNA barcoding is widely used across families (e.g. Hausmann et al. 2011; Lukhtanov et al. 2025), although it may be less effective in cases of recent speciation or where multilocus markers better reflect species boundaries.

Recently, it has been shown that the geometrid moth genus *Nychiodes* Lederer, 1853 contains species with high intraspecific variation in external and internal genitalia characters, which hamper their identification (Wanke et al. 2020). This genus consists of 25 species distributed from Western Europe and North Africa to Iran, Afghanistan and Pakistan (Müller et al. 2019; Wanke et al. 2020; Rajaei et al. 2023). Within this genus, a comprehensive examination of numerous specimens, including their genitalia, has shown that most species occur in the Middle East (Wanke et al. 2020). Larvae of *Nychiodes* species are characterized by an orange collar, while their adults are distinguished by prominent ante- and postmedial lines on the forewings (Müller et al. 2019; Wanke et al. 2021). *Nychiodes divergaria* Staudinger, 1892, shows significant intraspecific variation in both wing pattern and male genitalia morphology, challenging its delimitation where it is sympatric with its sister species. This species is distributed from southern Turkey and southern Armenia along the Zagros Mountains in Iran to the border of Pakistan and along the Alborz mountains in northern Iran (Wanke et al. 2020; Fig. 1). In southern Iran, *N. divergaria* is sympatric with its sister species, the endemic *N. subvirida* Brandt, 1938 (Wanke et al. 2020; Fig. 1). However, the female genitalia show clear diagnostic characters for species

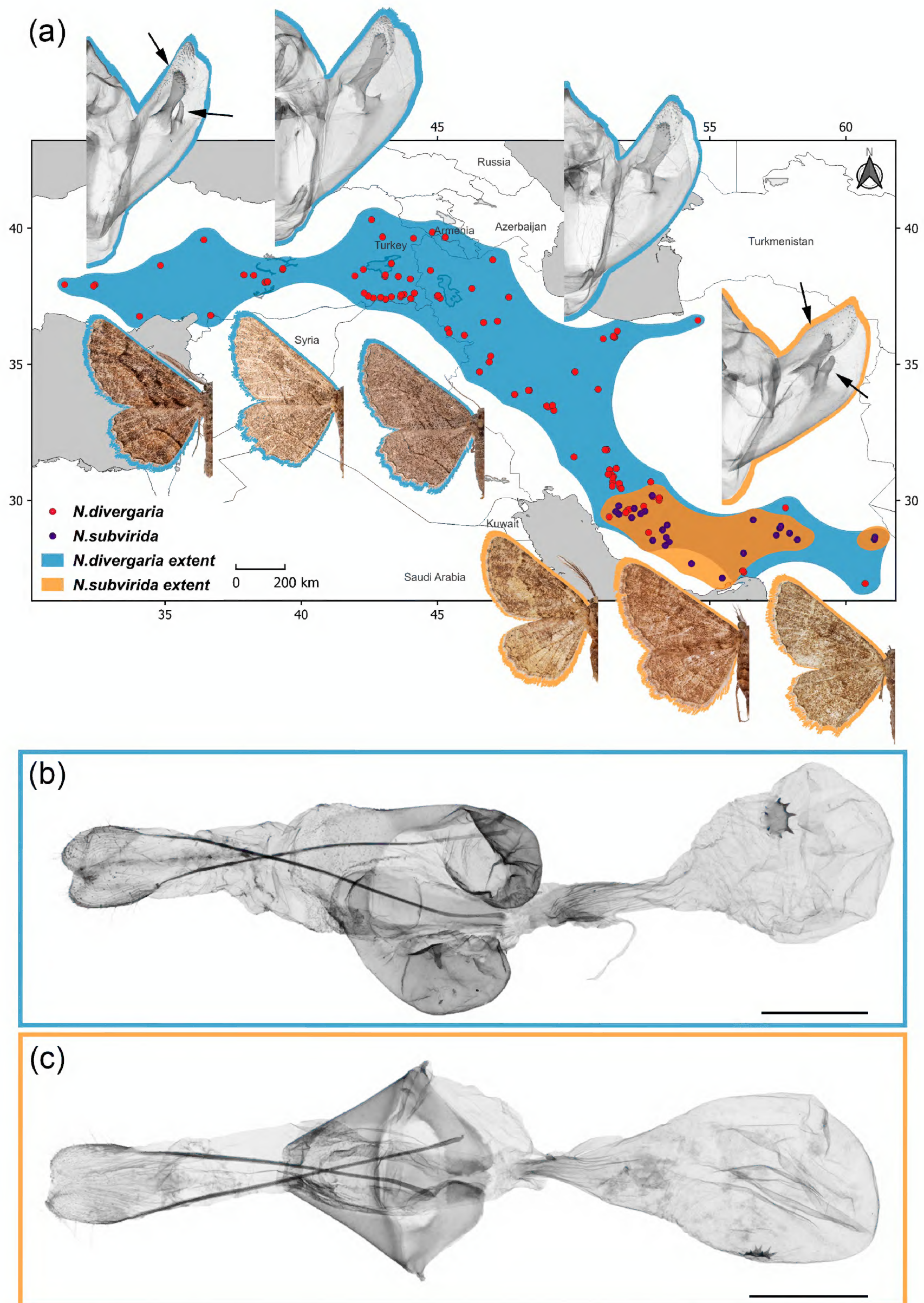


Figure 1. Distribution ranges and morphological characters of the studied *Nychiodes* species, *N. divergaria* and *N. subvirida*. **a.** Examined records and extrapolated distribution ranges. Wing pattern and the male genitalia capsule are marked with blue outlines for *N. divergaria* and with orange outlines for *N. subvirida*. Arrows on the male genitalia capsule indicate structural variability in details of valva. Distribution ranges were generated by a buffer of 50 km around examined records; **b.** Female genitalia of *N. divergaria*; **c.** Female genitalia of *N. subvirida*. Wing and male genitalia photographs are not to the same scale. Scale bars: 1 mm (**b, c**).

delimitation, with two sclerotized spherical patches in *N. divergaria* that are not present in *N. subvirida* (Fig. 1), whereas COI barcodes are intermixed, suggesting either recent speciation with ILS or mitochondrial introgression across species boundaries (Wanke et al. 2020).

To robustly delimit these taxonomically challenging species, we employed complementary approaches. First, we analyzed distribution data to test the null hypothesis that the putative species share the same ecological niche, with the alternative hypothesis that niche divergence facilitated species formation. Second, we used a multispecies coalescent model of independent nuclear genes to test the monophyly of the putative species. Together, these approaches evaluate whether morphological and genetic polymorphism reflects recent speciation events.

Material and methods

Occurrence data

We compiled the current distribution of *Nychiodes divergaria* and *N. subvirida* across their respective ranges. Our dataset includes 126 occurrences for *N. divergaria* and 31 for *N. subvirida*, identified using morphology and DNA barcodes from the study by Wanke et al. (2020), which are in strong concordance. After removing duplicated and missing values for longitude and latitude, we finalized the dataset with 110 occurrences for *N. divergaria* and 27 for *N. subvirida*.

Variable selection

We tested 19 high-resolution bioclimatic variables from the Chelsa dataset (approximately $1 \times 1 \text{ km}^2$; Karger et al. 2017) to identify variables with higher contribution and minimal multicollinearity issues using various analyses. First, using pairwise correlation tests ($r > 0.75$), we investigated the correlation among different variables using `cor` function from `corrplate` package (v. 0.92; Wei and Simko 2021). Then we shortlisted the variables with minimum collinearity issues using the variation inflation factor (VIF) from `usdm` package (Naimi et al. 2014). Finally, we checked the contribution of each variable across the climate space by principle component analysis (PCA) using `dudi.pca` function from `ade4` R package (Dray and Dufour 2007; Noori et al. 2024a, 2025; R Core Team 2024; Suppl. material 1: fig. S1). We selected seven variables for *N. divergaria* and six for *N. subvirida* (Table 1; for more details see the Suppl. material 1: fig. S1).

Species distribution models (SDMs)

Using occurrence data and selected environmental covariates, we modeled the current habitat suitability for the studied species. Following previous studies (Ginal et al. 2022; Noori et al. 2024a, 2024b, 2025), we modeled species distributions using the maximum-entropy algorithm (MaxEnt) implemented through the `biomod2` package in R (R Core Team 2024; Thuiller et al. 2024). MaxEnt has been widely used for modeling species habitat suitability, particularly in the case of presence-only data (Phillips et al. 2009; Fourcade et al. 2014; Noori et al. 2024b; Yang et al. 2024).

Table 1. List of selected variables for the studied species of *Nychiodes*.

Species	Selected variables
<i>N. divergaria</i>	Bio3: Isothermality Bio7: Temperature annual range Bio8: Mean temperature of wettest quarter Bio9: Mean temperature of driest quarter Bio12: Annual precipitation Bio14: Precipitation of driest month Bio15: Precipitation seasonality (coefficient of variation)
<i>N. subvirida</i>	Bio3: Isothermality Bio7: Temperature annual range Bio9: Mean temperature of driest quarter Bio14: Precipitation of driest month Bio15: Precipitation seasonality (coefficient of variation) Bio16: Precipitation of wettest quarter

However, in our analysis, we implemented several strategies to reduce the uncertainty and potential biases (see SM for more details). First, 10,000 background/pseudo-absence points were randomly sampled across the accessible area for the studied species. Next, we constructed ensemble final models by selecting models with higher performance across 100 replicates, as outlined by Noori et al. (2024b, 2025). To do so, we only used the model with higher values of area under the ROC Curve ($AUC_{test} > 0.7$). The resulting habitat suitability maps were subsequently thresholded by applying a 10% omission rate. Finally, using a multivariate environmental similarity surface (MESS) from dismo package, we defined the areas within and outside of the training range (Suppl. material 1: figs S2, S3; Elith et al. 2010; Rödder et al. 2013; Ginal et al. 2022).

Niche and hypervolume assessment

The occupied climate space and hypervolume of the *Nychiodes* species were assessed to define the similarity/dissimilarity volume and overlaps of their niches based on SDM results (Fig. 2, Table 3). The resulting SDMs habitat suitability of the species were masked by a Minimum Convex Polygon (MCP) based on a buffer of 50 km around the species occurrences using rangeBuilder package (v. 2.1; Davis Rabosky et al. 2016). Using two thresholds of 0.25 and 0.5, a binary presence-absence raster was generated. Next, 1,000 coordinated points were randomly selected across the species range. The climate values of these points were extracted from the shared bioclimatic variables between species (bio3, 7, 9, 14, and 15). This dataset was used to reduce the multidimension of the climate space using PCA for further steps (detailed in SM).

Niches of both species were created using ecospat.grid.clim.dyn function of ecospat package based on the first two PCA components (v. 4.1.1; Di Cola et al. 2017; Broennimann et al. 2024). Niche overlaps were assessed using two indices for similarity (Hellinger’s I (I)) and dissimilarity (Schoener’s D (D)) of species niches using ecospat.niche.overlap function (Broennimann et al. 2024). These metrics indicate the similarity (I) and dissimilarity (D) of niches between species (Di Cola et al. 2017; Table 3). Then, we performed a non-parametric Wilcoxon test to determine if species niches between the two species significantly differ (Patil 2021).

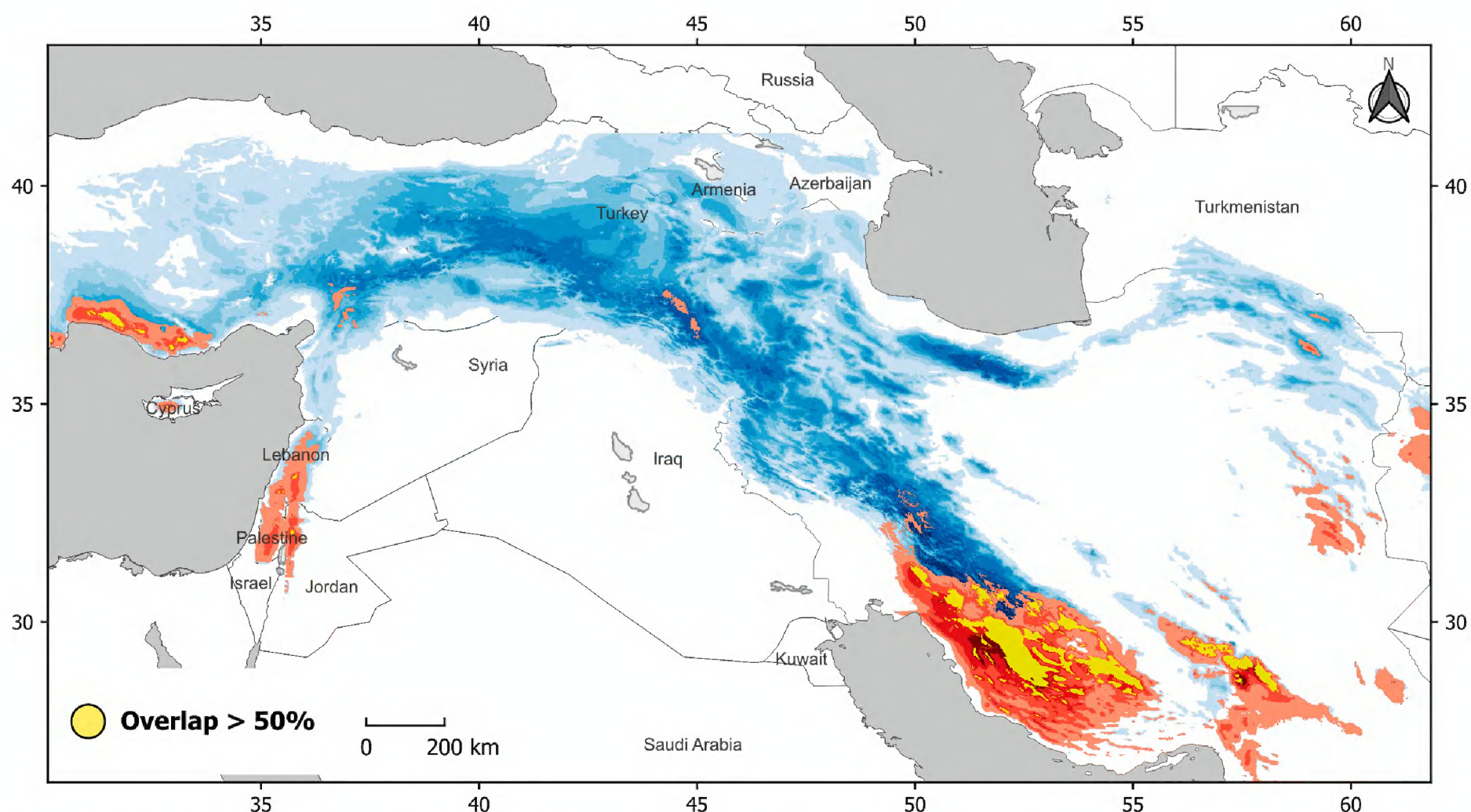


Figure 2. A map for habitat suitability of *N. divergaria* (blue) and *N. subvirida* (red). The white area show the unsuitable habitats for both species. Yellow color indicates overlapped areas with higher habitat suitability (>50%) between two species.

Finally, we quantified the occupied climatic hypervolume of each species and their degree of (dis)similarity using the first two principal component (PC) axes. Species hypervolumes were estimated using the `hypervolume_gaussian` function from the `hypervolume` package (v. 3.1.6; Blonder et al. 2025), based on 1,000 stochastic samples per species. We then quantified hypervolume size, intersection, and similarity between species using Jaccard and Sørensen indices at both suitability thresholds (Blonder et al. 2025; Table 3).

Molecular data generation

DNA extraction and amplification of the barcode fragment (658 base pairs of the 5' terminus) of the mitochondrial cytochrome c oxidase subunit I (COI) were conducted following the manufacturer's protocol of the DNeasy Blood and Tissue kits (Qiagen, Hilden, Germany) using a leg from a single dry collection specimen in the framework of the revision of the genus *Nychiodes* (Wanke et al. 2020). Amplification of nuclear markers was conducted following Wahlberg and Wheat (2008) and Wahlberg et al. (2016) using the above-mentioned extracts. We amplified and sequenced five protein-coding nuclear gene regions for downstream analyses: arginine kinase (ArgK), sarco/endoplasmic reticulum calcium ATPase (Ca-ATPase), elongation factor 1 alpha (EF-1alpha), ribosomal protein (RpS5) and wingless (wgl) for downstream analyses. Sequence alignments for each gene were performed using the Muscle algorithms as implemented in MEGA11 (Tamura et al. 2021). Four sister species (*Nychiodes leviata* Brandt, 1938, *N. farinosa* Brandt, 1938, *N. antiquaria* Staudinger, 1892, and *N. admirabila* Brandt, 1938) were used as outgroup for phylogenetic analysis. GenBank accession numbers for sequences used are presented in Suppl. material 1: table S2.

Phylogenetic analyses

For the maximum-likelihood (ML) analysis, the six concatenated gene sequences were analyzed using IQ-TREE v. 2.1.3 (Minh et al. 2020). The best partitioning scheme for each gene's codon position was identified with ModelFinder (Kalyaanamoorthy et al. 2017) and selected based on the corrected Akaike Information Criteria. We used the ultrafast bootstrap approximation method (UFBoot2) to assess branch support with 1,000 replicates (Minh et al. 2013). The best-fitting substitution models for each gene were as follows: COI, TPM2u+F+I; ArgK, HKY+F+I; Ca-ATPase, JC; EF-1alpha, TNe+I; RpS5, K2P+I; wgl, TN+F+I. The resulting trees were visualized and rooted to the outgroups mentioned above using FigTree v. 1.4.3 (Rambaut 2021) and further modified in CoreIDRAW v. 24.

In addition, to account for possible incomplete lineage sorting common in recent speciation events, a coalescent-based method was employed to estimate the species tree. Gene trees for four nuclear genes, excluding ArgK and Ca-ATPase genes due to large data omissions, were generated via IQ-TREE with 1,000 ultrafast bootstraps and ModelFinder. ASTRAL-III v. 5.7.8 (Zhang et al. 2018) was used to infer species trees for both data before and after the removal of recombinant loci from multi-individual dataset (Rabiee et al. 2019) with 100 replicates of multi-locus bootstrapping (Seo 2008).

Species delimitation (SD) analyses

We conducted the analyses using three complementary approaches: a distance-based method (ABGD) based on mtCOI and two multispecies coalescent methods (bPTP and GMYC) that incorporated multi-locus data, combining mtCOI with five nuclear gene data. Automatic barcode gap discovery (ABGD) analysis was performed under the K2P model. The parameter settings were as follows: $P_{\min} = 0.001$, $P_{\max} = 0.1$, steps = 10, relative gap width = 1.5, number of bins = 20, with all other settings at default. Both bPTP (Bayesian Poisson Tree Processes) and GMYC (Generalized Mixed Yule-Coalescent) require an ultrametric tree. To obtain this, we used the ML tree generated by IQ-TREE, and subsequently applied a molecular clock transformation via 'chronos' function in the R package ape (Paradis et al. 2004). The bPTP analysis was conducted using the online web server (<https://species.h-its.org/>) with the ultrametric tree as input. The Markov chain Monte Carlo (MCMC) chain was run for 200,000 generations, with a thinning interval of 1000, a burn-in of 20%, and a default random seed. Outgroup taxa were removed prior to analysis. The GMYC analysis was performed using the single-threshold model implemented in the R package splits (Ezard et al. 2009), with the ultrametric tree as input after outgroup removal.

Species network analysis using mtCOI

Relationships between haplotypes of *Nychiodes divergaria* and *N. subvirida* were assessed using a median-joining haplotype network created with PopArt v. 1.7.2 (Leigh and Bryant 2015) based on mtCOI sequences.

Results

Niche differentiation

Through our variable selection, distinct sets of climate variables were selected for each studied species of *Nychiodes* (Table 1). Five variables, which made significant contributions, are shared between the two species, while additionally variables are specific to each (Suppl. material 1: table S1, fig. S2), consistent with niche divergence between taxa. The contribution values of the climate variables in SDMs shows that the variables Bio7 (Temperature Annual Range) and Bio12 (Annual Precipitation) have the highest contribution for modeling *N. divergaria*, whereas Bio15 (Precipitation Seasonality) and Bio16 (Precipitation of Wettest Quarter) are most influential for *N. subvirida* (Table 2). Except for Bio3 and Bio9, the other shared variables between the two species with highest contribution are completely opposing each other. For example, Bio12, which has the highest contribution to models of *N. divergaria*, are exactly in opposition to this variable in *N. subvirida* (Suppl. material 1: fig. S1b, d). Similar patterns can be seen for Bio7 and Bio16. Overall, our findings indicate that *N. divergaria* occupies wetter niches across a broader climate niche space, while *N. subvirida* extends across drier and narrower niches.

The resulting ensemble models indicate differing habitat suitability across the distribution ranges and in the overlapping areas of the two species (Fig. 2, Suppl. material 1: figs S2, S3). For *N. divergaria*, regions of higher habitat suitability extend from southern Turkey, through the central Zagros and Alborz Mountain ranges. In contrast, *N. subvirida* is mainly restricted to the southern Zagros Mountain and some mountains in southeastern Iran (Suppl. material 1: figs S2, S3). Overall, in the overlapping areas (Fig. 2), *N. divergaria* predominantly occupied the eastern slopes of the Zagros Mountains, while *N. subvirida* is primarily distributed along the southern slopes of the Zagros Mountains, extending towards coastal areas of the Persian Gulf.

Niche overlap and hypervolume

As shown in Table 3, the niches (occupied climate spaces) of two species are significantly different at two habitat suitability thresholds, supporting the niche divergence hypothesis. The two species partially overlap in their climate

Table 2. The average values for TSS and AUC, and contribution of selected environmental variables, for *Nychiodes* species. The contributions of dominant climate variables to the model are displayed in bold.

Species	TSS	AUC	bio3	bio7	bio8	bio9	bio12	bio14	bio15	bio16
<i>N. divergaria</i>	0.67	0.91	6.70	20.1	0.159	8.28	23.4	3.65	11.0	—
<i>N. subvirida</i>	0.81	0.97	0.06	16.1	—	0.56	—	7.93	31.4	17.9

Table 3. Results of Wilcoxon test and Schoener’s *D* and Hellinger’s *I* using the first two PCA components for habitat (dis)similarity and the occupied hypervolume for each species and their similarity and intersection of *Nychiodes divergaria* and *N. subvirida*.

Threshold	<i>p</i> -value	<i>D</i>	<i>I</i>	<i>N. divergaria</i> volume	<i>N. subvirida</i> volume	Jaccard	Sørensen	Intersection
>50%	<0.001***	0.38	0.61	28.71	12.44	0.33	0.49	10.12
>25%	<0.001***	0.33	0.57	30.02	10.31	0.30	0.47	9.39

spaces across Zagros Mountain (Fig. 2), where *N. divergaria* occupies a broader climate niche space compared with *N. subvirida* (see Suppl. material 1: figs S5–S12, for more details).

Genetic data

The ML tree based on the six concatenated gene dataset (mitochondrial COI and nuclear five genes) supports the *N. divergaria*–*subvirida* complex as a monophyletic group with 100% bootstrap support (Fig. 3a). Within this lineage, the two species are intermixed, likely reflecting discordance between mitochondrial and nuclear signals in the concatenated analysis. In contrast, the species tree constructed using ASTRAL based exclusively on four nu-

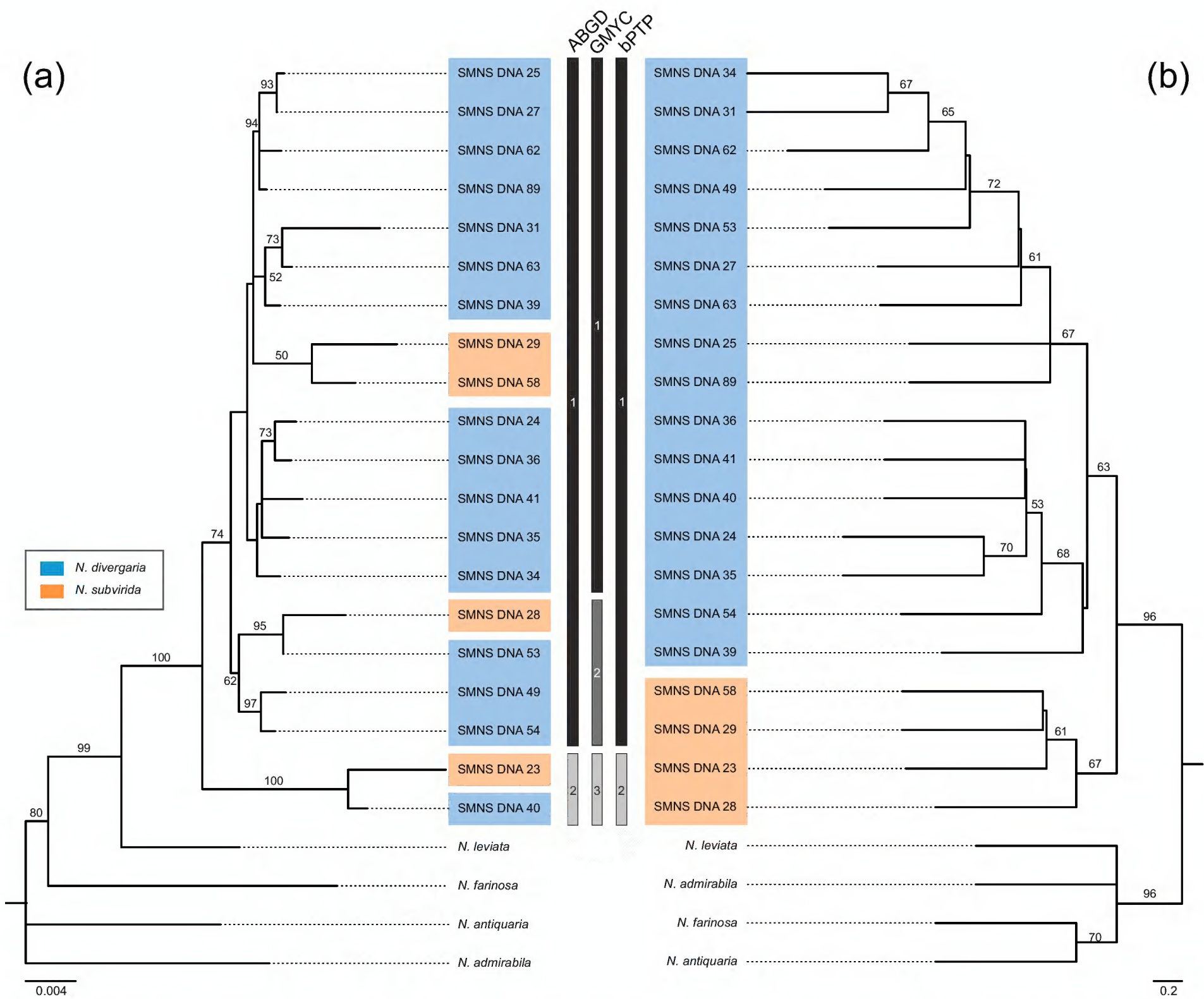


Figure 3. Phylogenetic relationships and species delimitation (SD) within the *Nychiodes divergaria*–*subvirida* complex based on a multi-individual dataset. **a.** Maximum-likelihood (ML) tree inferred with IQ-TREE from a concatenated alignment including COI and five nuclear gene sequences; **b.** Species tree inferred under the multispecies coalescent model using ASTRAL, based exclusively on genes from four nuclear loci (ArgK and Ca-ATPase excluded due to missing data). The middle panel presents SD analyses based on ABGD (COI only) and GMYC and bPTP (multilocus dataset). Species delimitation bars correspond to the individuals shown in the ML tree (left panel). Each bar reflects the number of delimited species (indicated by numbers) and the corresponding grouping of individuals.

clear loci clearly separates the two species (Fig. 3b), though with moderate bootstrap support (63% for *N. divergaria* and 67% for *N. subvirida*). The *N. divergaria*–*subvirida* lineage remains strongly supported in the multispecies coalescent tree inferred with ASTRAL, with 96% bootstrap support. The four sister species—*N. leviata*, *N. farinosa*, *N. antiquaria*, and *N. admirabila*—are clearly separated from the *N. divergaria*–*subvirida* lineage and served as out-groups in both the ML and coalescent-based analyses.

The current classification of the *Nychiodes divergaria*–*subvirida* complex, based on morphological traits, was not supported by molecular species delimitation (SD) analyses. Instead, SD analyses suggested completely different groupings of individuals. Both ABGD (based on mitochondrial COI) and bPTP (based on multigene data) identify the same two groups with identical individual compositions, whereas GMYC (also based on multigene data) further subdivided one of these groups (Fig. 3, middle bars).

N. divergaria and *N. subvirida* share COI haplotypes and show high external morphological variation, but male genitalia and nuclear DNA support their distinction. Ecologically, *divergaria* mostly overlaps with *subvirida*, indicating limited niche divergence.

Discussion

The present study highlights the importance of integrative taxonomic approaches to understand species differentiation in recent speciation events. *Nychiodes divergaria* and *N. subvirida* share COI haplotypes and show high external and internal (male genitalia) morphological variation, but female genitalia (see Wanke et al. 2020) and nuclear DNA support their distinction. Ecologically, *N. divergaria* and *N. subvirida* show a considerable geographic overlap, particularly at higher elevations, indicating that spatial co-occurrence alone does not necessarily reflect ecological similarity. COI discordance likely reflects incomplete lineage sorting or recent introgression, which is expected in recently diverged species pairs.

The niche qualification test revealed significant ecological niche divergence between the two species (Table 3). While temperature- and precipitation-related variables jointly influence the distribution of *N. divergaria*, the distribution of *N. subvirida* is mainly determined by precipitation-related variables (Table 2). In addition, opposite directions of PCA loadings of climate variables indicate that the species occupy distinct climate niche spaces (Suppl. material 1: figs S2, S3), despite their partial geographic overlap. A possible interpretation would be that *N. divergaria* is associated with relatively wetter climatic conditions, whereas *N. subvirida* tends to occur in comparatively drier environments (Suppl. material 1: fig. S1).

This ecological differentiation is likely linked to habitat and bioclimatic region differentiation across the Zagros Mountains, a major biogeographic transition zone in this region. The southern Zagros, with a hotter and drier climate, is located at a transition zone between Mediterranean and subtropical macroclimate regions along the northern costal of the Persian Gulf. On the other hand, north-western slopes of Zagros Mountain have more Mediterranean climate condition with higher precipitation (Djamali et al. 2011). Furthermore, the overlapping areas between the two species extend across two different ecoregions: Zagros Mountains Forest steppe and Nubo-Sindian desert and semi-deserts (Fig. 2; Dinerstein et al. 2017; Noori et al. 2025). Accordingly, although the two species may co-occur

at similar elevations, they occupy different ecological niches, with *N. divergaria* predominantly associated with the eastern slopes of the Zagros Mountains and *N. subvirida* occurring mainly on the western and southern slopes of the mountain ranges. Previous studies have shown that Zagros Mountains serves as a biogeographic barrier and as a corridor at the intersection of two biogeographical realms, the Palearctic and the Saharo-Arabian, which has dramatically shaped the biodiversity patterns in western Asia (Noori et al. 2024c, 2025). The observed niche differentiation between *N. divergaria* and *N. subvirida* likely reflects this complex role of the Zagros Mountains as a dynamic transition zone rather than a strict dispersal barrier. The constructed species tree, based on four nuclear loci, clearly separates *N. divergaria* and *N. subvirida*, albeit with a moderate bootstrap support. Species delimitation remains challenging in recently diverged taxa due to discrepancies among different datasets and analytical methods.

While traditional taxonomy based on external and genitalia morphology (Wanke et al. 2020) and analyses of nuclear markers confirm two distinct lineages, COI data show discordance, which more likely reflect mitochondrial introgression, and are therefore misleading in delimiting species.

Notably, characters of the female genitalia aligned well with the nuclear species tree inferred under the multispecies coalescent framework, that incorporates discordance among gene trees caused by likely introgression. The finding suggests that female genitalia represent a relatively conserved and reliable morphological trait for species delimitation in this group. The sharing of mitochondrial lineages between *N. divergaria* and *N. subvirida* likely reflects mitochondrial introgression, rather than evidence against species-level separation.

However, multispecies coalescent method, such as GMYC, should be interpreted with caution, as they have a known tendency to oversplit species (Dellicour and Flot 2018). Similar patterns have been reported in other insect groups, such as recently diverged grasshopper lineages (Nolen et al. 2020), indicating that this issue extends beyond our focal group and represents a broader challenge in species delimitation across insects. These inconsistencies highlight the limitations of relying solely on mitochondrial or limited nuclear data and emphasize the importance of integrating multiple lines of evidence, including morphology, distribution, ecology, and broader genomic data, to achieve robust species boundaries. Given the observed conflicts, which are relatively common in many insect taxa, future studies incorporating additional nuclear markers or genome-wide data may provide further insights into species limits and the complex evolutionary dynamics of this group. Considering all available data, including areas of conflict among datasets, we follow the principles of integrative taxonomy (Padial et al. 2010) and regard *N. divergaria* and *N. subvirida* as two separate species. Here we point out the challenges inherent in species delimitation in a case of recent divergence and different type of data allow for different interpretations. Despite the significant intraspecific morphological variation, female genitalia remain a reliable diagnostic trait for defining species boundaries in this genus (see Wanke et al. 2020). Furthermore, although the two species partially share their geographic range, they occupy distinct ecological niches shaped by climatic differentiation across the Zagros Mountains. The transitional zone between Mediterranean and tropical macroclimate appears to have played a crucial role in driving ecological divergence, thereby contributing to the evolutionary trajectories and today's distributions of the two species (Noori et al. 2024c). This study provides further evidence that incongruence among data-

sets is an expected outcome in young species pairs and demonstrates that multi-species coalescent approaches, when interpreted in an integrative framework, are valuable tools for clarifying species boundaries in emerging species.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

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Use of AI

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Author contributions

Dominic Wanke: Conceptualization, data curation, formal analysis, investigation, methodology, project administration, validation, visualization, writing – original draft, writing – review & editing. Simon Müller: Validation, writing – original draft, writing – review & editing. Sajad Noori: Conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing – original draft, writing – review & editing. Ricardo J. Pereira: Conceptualization, methodology, validation, writing – original draft, writing – review & editing. Hossein Rajaei: Resources, validation, writing – original draft, writing – review & editing. Pasi Sihvonen: Conceptualization, validation, writing – original draft, writing – review & editing. Kyung Min Lee: Conceptualization, data curation, formal analysis, investigation, methodology, project administration, validation, visualization, writing – original draft, writing – review & editing.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

References

- Antil S, Abraham JS, Sripoorna S, Maurya S, Dagar J, Makhija S, Bhagat P, Gupta R, Sood U, Lal R, Toteja R (2023) DNA barcoding, an effective tool for species identification: A review. *Molecular Biology Reports* 50(1): 761–775. <https://doi.org/10.1007/s11033-022-08015-7>

- Blonder B, Morrow CB, Brown S, Butruille G, Chen D, Laini A, Harris DJ, Violet C (2025) hypervolume: High Dimensional Geometry, Set Operations, Projection, and Inference Using Kernel Density Estimation, Support Vector Machines, and Convex Hulls. R package version 3.1.6. <https://doi.org/10.32614/cran.package.hypervolume>
- Broennimann O, Di Cola V, Guisan A (2024) Ecospat: Spatial Ecology Miscellaneous Methods. R package version 4.1.1. <https://doi.org/10.32614/cran.package.ecospat>
- Cook LG, Edwards RD, Crisp MD, Hardy NB (2010) Need morphology always be required for new species descriptions? Invertebrate Systematics 24(3): 322–326. <https://doi.org/10.1071/IS10011>
- Davis Rabosky AR, Cox CL, Rabosky DL, Title PO, Holmes IA, Feldman A, McGuire JA (2016) Coral snakes predict the evolution of mimicry across New World snakes. Nature Communications 7(1): 11484. <https://doi.org/10.1038/ncomms11484>
- Degnan JH, Rosenberg NA (2009) Gene tree discordance, phylogenetic inference, and the multispecies coalescent. Trends in Ecology & Evolution 24(6): 332–340. <https://doi.org/10.1016/j.tree.2009.01.009>
- Dellicour S, Flot JF (2018) The hitchhiker’s guide to single-locus species delimitation. Molecular Ecology Resources 18(6): 1234–1246. <https://doi.org/10.1111/1755-0998.12908>
- Di Cola V, Broennimann O, Petitpierre B, Breiner FT, d’Amen M, Randin C, Engler R, Pottier J, Pio D, Dubuis A, Pellissier L, Mateo RG, Hordijk W, Salamin N, Guisan A (2017) eco-spat: An R package to support spatial analyses and modeling of species niches and distributions. Ecography 40(6): 774–787. <https://doi.org/10.1111/ecog.02671>
- Dinerstein E, Olson D, Joshi A, Vynne C, Burgess ND, Wikramanayake E, Hahn N, Palminteri S, Hedao P, Noss R, Hansen M, Locke H, Ellis EC, Benjamin Jones B, Charles Victor Barber CV, Randy Hayes R, Cyril Kormos C, Vance Martin V, Crist E, Sechrest W, Price L, Baillie JEM, Weeden D, Suckling K, Davis C, Sizer N, Moore R, Thau D, Birch T, Potapov P, Turubanova S, Tyukavina A, De Souza N, Pintea L, Brito JC, Llewellyn OA, Anthony G, Miller AG, Annette Patzelt A, Shahina A, Ghazanfar SA, Timberlake J, Klöser H, Shennan-Farpón YS, Kindt R, Barnekow Lillesø JP, Van Breugel P, Graudal L, Vogé M, Al-Shammari KF, Saleem M (2017) An ecoregion-based approach to protecting half the terrestrial realm. Bioscience 67(6): 534–545. <https://doi.org/10.1093/biosci/bix014>
- Djamali M, Akhane H, Khoshravesh R, Andrieu-Ponel V, Ponel P, Brewer S (2011) Application of the global bioclimatic classification to Iran: Implications for understanding the modern vegetation and biogeography. Ecologia Mediterranea 37(1): 91–114. <https://doi.org/10.3406/ecmed.2011.1350>
- Dray S, Dufour A (2007) The ade4 package: Implementing the duality diagram for ecologists. Journal of Statistical Software 22(4): 1–20. <https://doi.org/10.18637/jss.v022.i04>
- Elith J, Kearney M, Phillips S (2010) The art of modelling range-shifting species. Methods in Ecology and Evolution 1(4): 330–342. <https://doi.org/10.1111/j.2041-210X.2010.00036.x>
- Ezard T, Fujisawa T, Barraclough TG (2009) Splits: species’ limits by threshold statistics. R package version 1(11): r29.
- Fourcade Y, Engler JO, Rödder D, Secondi J (2014) Mapping species distributions with MAXENT using a geographically biased sample of presence data: A performance assessment of methods for correcting sampling bias. PLoS ONE 9(5): e97122. <https://doi.org/10.1371/journal.pone.0097122>
- Ginal P, Tan WC, Rödder D (2022) Invasive risk assessment and expansion of the realized niche of the Oriental garden lizard *Calotes versicolor* species complex (Daudin, 1802). Frontiers of Biogeography 14(3): e54299. <https://doi.org/10.21425/F5FBG54299>

- Hardwick DF (1950) Preparation of slide mounts of lepidopterous genitalia. *Canadian Entomologist* 82(11): 231–235. <https://doi.org/10.4039/Ent82231-11>
- Hausmann A, Haszprunar G, Segerer AH, Speidel W, Behounek G, Hebert PDN (2011) Now DNA-barcoded: The butterflies and larger moths of Germany. *Spixiana* 34: 47–58.
- Hebert PD, Cywinska A, Ball SL, deWaard JR (2003a) Biological identifications through DNA barcodes. *Proceedings. Biological Sciences* 270(1512): 313–321. <https://doi.org/10.1098/rspb.2002.2218>
- Hebert PD, Ratnasingham S, deWaard JR (2003b) Barcoding animal life: Cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings. Biological Sciences* 270(Suppl.): S96–S99. <https://doi.org/10.1098/rsbl.2003.0025>
- Hench K, Helmkampf M, McMillan WO, Puebla O (2022) Rapid radiation in a highly diverse marine environment. *Proceedings of the National Academy of Sciences of the United States of America* 119(4): e2020457119. <https://doi.org/10.1073/pnas.2020457119>
- Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermiin LS (2017) ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nature Methods* 14(6): 587–589. <https://doi.org/10.1038/nmeth.4285>
- Karger DN, Conrad O, Böhrer J, Kawohl T, Kreft H, Soria-Auza RW, Zimmermann NW, Linder HP, Kessler M (2017) Climatologies at high resolution for the Earth's land surface areas. *Scientific Data* 4(1): 1–20. <https://doi.org/10.1038/sdata.2017.122>
- Kress WJ, Erickson DL (2008) DNA barcodes: Genes, genomics, and bioinformatics. *Proceedings of the National Academy of Sciences of the United States of America* 105(8): 2761–2762. <https://doi.org/10.1073/pnas.0800476105>
- Leigh JW, Bryant D (2015) popart: Full-feature software for haplotype network construction. *Methods in Ecology and Evolution* 6(9): 1110–1116. <https://doi.org/10.1111/2041-210X.12410>
- Lukhtanov VA, Makhov IA, Gagarina AV, Romanovich AE (2025) Taxonomy of the West Palaearctic butterfly genus *Palaeophilotes* Forster, 1938 (Lepidoptera: Lycaenidae) based on combined analysis of COI barcodes and multilocus nuclear markers. *Zootaxa* 5584(4): 570–580. <https://doi.org/10.11646/zootaxa.5584.4.8>
- Maddison WP (1997) Gene trees in species trees. *Systematic Biology* 46(3): 523–536. <https://doi.org/10.1093/sysbio/46.3.523>
- Meyer CP, Paulay G (2005) DNA barcoding: Error rates based on comprehensive sampling. *PLoS Biology* 3(12): e422. <https://doi.org/10.1371/journal.pbio.0030422>
- Minh BQ, Nguyen MAT, von Haeseler A (2013) Ultrafast Approximation for Phylogenetic Bootstrap. *Molecular Biology and Evolution* 30(5): 1188–1195. <https://doi.org/10.1093/molbev/mst024>
- Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, von Haeseler A, Lanfear R, Teeling E (2020) IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. *Molecular Biology and Evolution* 37(5): 1530–1534. <https://doi.org/10.1093/molbev/msaa015>
- Müller B, Erlacher S, Hausmann A, Rajaei H, Sihvonen P, Skou P (2019) Ennominae II. In: Hausmann A, Rajaei H, Sihvonen P, Skou P (Eds) *The Geometrid Moths of Europe* 6. Brill, Leiden, 1–906. <https://doi.org/10.1163/9789004387485>
- Naimi B, Hamm NA, Groen TA, Skidmore AK, Toxopeus AG (2014) Where is positional uncertainty a problem for species distribution modelling. *Ecography* 37(2): 191–203. <https://doi.org/10.1111/j.1600-0587.2013.00205.x>
- Nolen ZJ, Yildirim B, Irisarri I, Liu S, Groot C (2020) Historical isolation facilitates species radiation by sexual selection: Insights from *Chorthippus* grasshoppers. *Molecular Ecology* 29(24): 4985–5002. <https://doi.org/10.1111/mec.15695>

- Noori S, Hofmann A, Rödder D, Husemann M, Rajaei H (2024a) A window to the future: Effects of climate change on the distribution patterns of Iranian Zygaenidae and their host plants. *Biodiversity and Conservation* 33(2): 579–602. <https://doi.org/10.1007/s10531-023-02760-2>
- Noori S, Rödder D, Soofi M, Hawlitschek O, Husemann M, Rajaei H (2024b) Extensive mismatch between protected areas and biodiversity hotspots of Iranian Lepidoptera. *Insect Conservation and Diversity* 17(6): 938–952. <https://doi.org/10.1111/icad.12760>
- Noori S, Zahiri R, Yusefi GH, Rajabizadeh M, Hawlitschek O, Rakhshani E, Husemann M, Rajaei H (2024c) Patterns of zoological diversity in Iran—A review. *Diversity* 16(10): 621. <https://doi.org/10.3390/d16100621>
- Noori S, Rödder D, Yusefi GH, Hawlitschek O, Husemann M, Rajaei H (2025) Biogeographic patterns of Iranian Lepidoptera: A framework for conservation. *Diversity & Distributions* 31(3): e70010. <https://doi.org/10.1111/ddi.70010>
- Padial JM, Miralles A, De la Riva I, Vences M (2010) The integrative future of taxonomy. *Frontiers in Zoology* 7(1): 16. <https://doi.org/10.1186/1742-9994-7-16>
- Paradis E, Claude J, Strimmer K (2004) APE: Analyses of phylogenetics and evolution in R language. *Bioinformatics* 20(2): 289–290. <https://doi.org/10.1093/bioinformatics/btg412>
- Patil I (2021) Visualizations with statistical details: The ‘ggstatsplot’ approach. *Journal of Open Source Software* 6(61): 3167. <https://doi.org/10.21105/joss.03167>
- Phillips SJ, Dudík M, Elith J, Graham CH, Lehmann A, Leathwick J, Ferrier S (2009) Sample selection bias and presence-only distribution models: Implications for background and pseudo-absence data. *Ecological Applications: A Publication of the Ecological Society of America* 19(1): 181–197. <https://doi.org/10.1890/07-2153.1>
- Pierce FN (1914) The genitalia of the Group Geometridae of the Lepidoptera of the British Islands. Northern Publishing Co., Liverpool, 88 pp. <https://doi.org/10.5962/bhl.title.19606>
- R Core Team (2024) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna. <https://www.R-project.org/> <https://doi.org/10.32614/r.manuals>
- Rabiee M, Sayyari E, Mirarab S (2019) Multi-allele species reconstruction using ASTRAL. *Molecular Phylogenetics and Evolution* 130: 286–296. <https://doi.org/10.1016/j.ympev.2018.10.033>
- Rajaei H (2015) Integrative taxonomy, a new approach to answer the question in the biosystematics: A case study of the genus *Gnopharmia* (Geometridae). *Entomologie Heute* 27: 25–35.
- Rajaei H, Aarvik L, Arnscheid WR, Baldizzone G, Bartsch D, Bengtsson BÅ, Bidzilya O, Buchner P, Buchsbaum U, Buszko J, Dubatolov VV, Erlacher S, Esfandiari M, De Freina JJ, Gaedike R, Gyulai P, Hausmann A, Haxaire J, Hobern D, Hofmann A, Ignatev N, Kaila L, Kallies A, Keil T, Kiss Á, Kitching IJ, Kun A, László GM, Leraut G, Mally R, Matov A, Meineke JU, Melichar T, Mey W, Mironov V, Müller B, Naderi A, Nässig WA, Naumann S, Nazari V, Van Nieukerken EJ, Nuss M, Pöll N, Prozorov AM, Rabieh MM, Rákossy L, Rindoš M, Rota J, Rougerie R, Schintlmeister A, Shirvani A, Sihvonen P, Simonsen TJ, Sinev SYu, Skou P, Sobczyk T, Sohn JC, Tabell J, Tarmann G, Tokár Z, Trusch R, Varga Z, Volynkin AV, Wanke D, Yakovlev RV, Zahiri R, Zehzad P, Zeller HC, Zolotuhin VV, Karsholt O (2023) Catalogue of the Lepidoptera of Iran. In: Rajaei H, Karsholt O (Eds) *Lepidoptera Iranica*. Integrative Systematics 6(Special Issue): 121–459.

- Rambaut A (2021) FigTree v1.3.1. Institute of Evolutionary Biology, University of Edinburgh, Edinburgh. <http://tree.bio.ed.ac.uk/software/figtree/>
- Rödger D, Lawing AM, Flecks M, Ahmadzadeh F, Dambach J, Engler JO, Habel JC, Hartmann T, Hörnes D, Ihlow F, Schidelko K, Stiels D, Polly PD (2013) Evaluating the significance of paleophylogeographic species distribution models in reconstructing Quaternary range-shifts of Nearctic chelonians. PLoS ONE 8(10): e72855. <https://doi.org/10.1371/journal.pone.0072855>
- Rubioff D, Cameron S, Will K (2006) A Genomic Perspective on the Shortcomings of Mitochondrial DNA for “Barcoding” Identification. The Journal of Heredity 97(6): 581–594. <https://doi.org/10.1093/jhered/esl036>
- Seo TK (2008) Calculating bootstrap probabilities of phylogeny using multilocus sequence data. Molecular Biology and Evolution 25(5): 960–971. <https://doi.org/10.1093/molbev/msn043>
- Sihvonen P (2001) Everted vesicae of the *Timandra griseata* group: Methodology and differential features (Geometridae, Sterrhinae). Nota Lepidopterologica 24: 57–63. <https://doi.org/10.5281/zenodo.16575262>
- Smith KL, Harmon LJ, Shoo LP, Melville J (2011) Evidence of constrained phenotypic evolution in a cryptic species complex of agamid lizards. Evolution; International Journal of Organic Evolution 65(4): 976–992. <https://doi.org/10.1111/j.1558-5646.2010.01211.x>
- Song H, Buhay JE, Whiting MF, Crandall KA (2008) Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. Proceedings of the National Academy of Sciences of the United States of America 105(36): 13486–13491. <https://doi.org/10.1073/pnas.0803076105>
- Tamura K, Stecher G, Kumar S (2021) MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Molecular Biology and Evolution 38(7): 3022–3027. <https://doi.org/10.1093/molbev/msab120>
- Thuiller W, Georges D, Gueguen M, Engler R, Breiner F, Lefourcade B, Patin R, Blancheteau H (2024) biomod2: Ensemble Platform for Species Distribution Modeling. R package version 4.2-5-2. <https://CRAN.R-project.org/package=biomod2>
- Wahlberg N, Wheat CW (2008) Genomic outposts serve the phylogenomic pioneers: Designing novel nuclear markers for genomic DNA extractions of Lepidoptera. Systematic Biology 57(2): 231–242. <https://doi.org/10.1080/10635150802033006>
- Wahlberg N, Peña C, Ahola M, Wheat CW, Rota J (2016) PCR primers for 30 novel gene regions in the nuclear genomes of lepidoptera. ZooKeys 596: 129–141. <https://doi.org/10.3897/zookeys.596.8399>
- Wanke D, Hausmann A, Krogmann L, Petrányi G, Rajaei H (2020) Taxonomic revision of the genus *Nychiodes* Lederer, 1853 (Geometridae: Ennominae: Boarmiini) with description of three new species—an integrative approach. Zootaxa 4812(1): 001–061. <https://doi.org/10.11646/zootaxa.4812.1.1>
- Wanke D, Leipnitz M, Rajaei H (2021) First captive rearing of the Iranian endemic *Nychiodes subvirida* Brandt, 1938 (Geometridae: Ennominae, Boarmiini). Entomologische Zeitschrift 131(2): 123–125.
- Wei T, Simko VR (2021) R package ‘corrplot’: Visualization of a Correlation Matrix (Version 0.92). <https://doi.org/10.32614/cran.package.corrplot>
- Wipfler B, Pohl H, Yavorskaya MI, Beutel RG (2016) A review of methods for analysing insect structures—The role of morphology in the age of phylogenomics. Current Opinion in Insect Science 18: 60–68. <https://doi.org/10.1016/j.cois.2016.09.004>

- Yang CQ, Wang Y, Li XH, Li J, Yang B, Orr MC, Zhang AB (2024) Environmental niche models improve species identification in DNA barcoding. *Methods in Ecology and Evolution* 15(12): 2343–2358. <https://doi.org/10.1111/2041-210X.14440>
- Zhang C, Rabiee M, Sayyari E, Mirarab S (2018) ASTRAL-III: Polynomial time species tree reconstruction from partially resolved gene trees. *BMC Bioinformatics* 19(S6): 153. <https://doi.org/10.1186/s12859-018-2129-y>

Supplementary material 1

Additional analyses

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Data type: docx

Explanation note: Supplementary material to Integration of morphologic and genetic data clarifies the evolution and species boundaries in *Nychiodes* Lederer, 1853 (Lepidoptera: Geometridae).

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